cDNA microarray analysis of differential gene expression and regulation in clinically drug-resistant isolates of *Candida albicans* from bone marrow transplanted patients

Zheng Xu[a], Li-Xin Zhang[b], Jun-Dong Zhang[a], Yong-Bing Cao[a], Yuan-Yuan Yu[a], De-Jun Wang[a], Kang Ying[c], Wan-Sheng Chen[a], Yuan-Ying Jiang[a,*]

[a]*Department of Pharmacology, School of Pharmacy, Second Military Medical University, 325 Guohe Road, Shanghai, 200433, P.R. China*
[b]*Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences, 510663, P.R. China*
[c]*State Key Laboratory of Genetic Engineering, Institute of Genetics, School of Life Science, Fudan University, Shanghai, 200433, P.R. China*

Abstract

Fungi have emerged as the fourth most common pathogens isolated in nosocomial bloodstream infections, and *Candida albicans* is the most common human fungal pathogen. Only a few antibiotics are effective in the treatment of fungal infections. In addition, the repetition and lengthy duration of fluconazole therapy has led to an increased incidence of azole resistance and treatment failure associated with *C. albicans*. To investigate the mechanism of drug resistance and explore new targets to treat clinically resistant fungal pathogens, we examined the large-scale gene expression profile of two sets of matched fluconazole-susceptible and -resistant bloodstream *C. albicans* isolates from bone marrow transplanted (BMT) patients for the first time by microarray analysis. More than 198 differentially expressed genes were identified and they were confirmed and validated by RT-PCR independently. Not surprisingly, the resistant phenotype is associated with increased expression of CDR mRNA, as well as some common genes involved in drug resistance such as *CaIFU5*, *CaRTA2* and *CaIFD6*. Meanwhile, some special functional groups of genes, including ATP binding cassette (ABC) transporter genes (*IPF7530*, *CaYOR1*, *CaPXA1*), oxidative stress response genes (*CaALD5*, *CaGRP1*, *CaSOD2*, *IPF10565*), copper transport and iron mobilization-related genes (*CaCRD1/2*, *CaCTR1/2*, *CaCCC2*, *CaFET3*) were found to be differentially expressed in the resistant isolates. Furthermore, among these differentially expressed genes, some co-regulated with *CaCDR1*, *CaCDR2* and *CaIFU5*, such as *CaPDR16* and *CaIFD6*, have a DRE-like element and may interact with *TAC1* in the promoter region. These findings may shed light on mechanisms of azole resistance in *C. albicans* and clinical antifungal therapy.

Keywords: *Candida albicans*; Microarray; Drug resistance; Bone marrow transplant; Differential gene expression

Introduction

Candida albicans is an important opportunistic fungal pathogen of humans and the major cause of

*Corresponding author. Tel.: +86 21 2507 0371.
E-mail address: jiangyy@smmu.edu.cn (Y.-Y. Jiang).

oropharyngeal candidiasis (OPC) in AIDS patients (Franz et al., 1998; Jones et al., 2004). Fluconazole, an azole antifungal agent, is widely used to treat OPC (Demain and Zhang, 2005). Due to prolonged and repeated use of this agent, emergence of azole-resistant *C. albicans* strains leading to treatment failures were observed (Morschhäuser, 2002; Rex et al., 1995; White et al., 2002). Thus it is desirable to explore and identify new targets for the treatment of clinically resistant fungal pathogens (Quan et al., 2006; Zhang, 2005; Zhang et al., 2005). Phenotypically stable resistance to azole antifungal agents in *C. albicans* can result from mutations or increased expression of genes involved in the ergosterol synthesis pathway (including the target enzyme 14-α demethylase), and increased expression of ATP-binding cassette (ABC) transporter and major facilitator efflux pumps (White et al., 1998). Moreover, the resistance can be rapidly developed in *C. albicans* after short exposures to the drug, both in vitro (Calvet et al., 1997) and in vivo (Marr et al., 1997; Nolte et al., 1997), notably in a strain of *C. albicans* that caused disseminated infection in a patient after bone marrow transplantation (BMT) (Marr et al., 1997).

Characterizing whole-genome expression using DNA microarrays provides a snapshot of an organism's genome in action by revealing the relative transcript levels of thousands of genes at a time (Liu et al., 2005). Previous studies on fluconazole resistance in *C. albicans* by microarray analysis have used either laboratory-derived azole-resistant strains, or clinical isolates obtained in the setting of OPC in patients with AIDS (Barker et al., 2004; Cowen et al., 2002; Rogers and Barker, 2002, 2003). There are important differences between the development of resistance in the setting of OPC as compared to that in candidemia in BMT patients. Resistance in OPC usually follows multiple treatment failures and dose escalations with a prolonged period of time. Azole-resistant isolates obtained from BMT patients showed a more rapid development of resistance and it occurred under conditions of much higher azole concentrations (Marr et al., 1998, 2001).

To investigate the mechanism of drug resistance, we examined the changes in a large-scale gene expression profile of two sets of matched clinical fluconazole susceptible and resistant *C. albicans* isolates from BMT patients.

Materials and methods

C. albicans isolates and growth conditions

The azole-susceptible strain SC5314 and two matched sets of susceptible and resistant isolates of *C. albicans* used in this study are listed in Table 1. FH1/FH5 and TL1/TL3 were colonizing and bloodstream isolates obtained from two different patients who underwent bone marrow transplant (BMT) operation, respectively. The two sets of isogenic *C. albicans* isolates were obtained from Theodore C. White and have been identified at Fred Hutchinson Cancer Research Center and described previously (Marr et al., 1997, 1998, 2001). FH1/FH5 and TL1/TL3 represent the same strain by the identical banding patterns of RFLP analysis, respectively. An aliquot of glycerol stock from each isolate was diluted in YPD broth (1% yeast extract, 2% peptone, 1% dextrose) and grown overnight at 30 °C in a shaking incubator.

Antifungal agents and IC80 determinations

Powder formulations of fluconazole (Roerig-Pfizer, New York, N.Y.) suspended in distilled water were made to a final concentration of 0.125 μg/ml, filter sterilized, and stored at −70 °C. Media utilized in these studies included YPD broth and RPMI 1640 with 0.165 M MOPS (morpholinopropanesulfonic acid) buffered at pH 7.0. Antifungal susceptibility testing was performed by the standardized microdilution method from NCCLS document M27-A (National Committee for Clinical Laboratory Standards, Wayne, PA). To ensure consistent results, 80% inhibitory concentrations

Table 1. Compilation of previously reported and verified IC80s and described resistance mechanisms for *C. albicans* isolates used in this study

Isolate	IC80 (μg/ml)		Previously described resistance mechanism(s)[a]
	Previously determined[a]	This study	
SC5314	<1	0.25	Laboratory control strain
TL1	1	1	
TL3	32	64	Overexpression of CDR genes
FH1	4	4	
FH5	>64	32	Overexpression of *CDR1* and *CDR2*

[a]As reported in references Marr et al. (1997, 1998, 2001).

(IC80s) were determined and compared under the same environmental conditions.

Microarray preparation

The microarray (3136 chip; United Gene Holdings, Ltd., China) used in our study consisted of 3136 sequences including full-length and partial complementary DNAs (cDNAs). Except for some control spots, the 3136 dotted array represented 3096 unigenes which were clustered from 11,032 total cDNAs by sequencing and bioinformatics analysis. Most of the genes were obtained by sequencing the clones isolated from a high ratio full-length cDNA library of *C. albicans* SC5314 using switching mechanism at 5′ end of RNA transcript (SMART) carried out at United Gene Holdings (Xu et al., 2005). In addition, the array included other known genes involved in drug resistance, drug targets and biofilm formation which were selected from NCBI Unigene set and cloned into plasmid vectors. The control spots were human glycerol-3-phosphate dehydrogenase (8 spots), actin genes (8 spots), and spotting solution alone without DNA (16 spots).

The 3136 chip was constructed according to our previously published protocol (Li et al., 2002; Cao et al., 2005). In brief, the cDNA inserts were amplified by use of the polymerase chain reaction (PCR) with universal primers and then purified with isopropanol precipitation. All PCR products were examined by agarose gel electrophoresis to ensure the quality and the identity of the amplified clones. The amplified PCR products were dissolved in a buffer containing $3 \times$ SSC solution ($1 \times$ SSC: 15 mM sodium citrate, pH 7.0, 150 mM NaCl). The above solutions were spotted onto sialylated slides (CEL Associates, Houston, TX) using a Cartesian PixSys 7500 motion control robot (Cartesian Technologies, Irvine, CA) fitted with ChipMaker Micro-Spotting Technology (TeleChem International, Sunnyvale, CA). Glass slides with spotted cDNA were then hydrated for 2 h in 70% humidity, dried for 0.5 h at room temperature, and UV cross linked (65 mJ/cm^2). They were further processed at room temperature by soaking them in 0.2% sodium dodecyl sulfate (SDS) for 10 min, followed by distilled H_2O for 10 min, and 0.2% sodium borohydride ($NaBH_4$) for 10 min.The slides were then dried before use.

RNA preparation and probe labeling

For transcriptional profiling, three independent replicates of each sample (Table 1) were grown overnight from the frozen archives in 50 ml YPD at 30 °C. Each independent replicate of cultures was diluted in 150 ml fresh YPD to an optical density at 600 nm (OD_{600}) of 0.1–0.2 and grown at 30 ° for 3 h to the early logarithmic phase of the density equivalent OD of 0.4–0.5 for subsequent RNA isolation. The cultures were harvested by centrifugation at 3600 *g* at 4 °C for 5 min. The medium was completely removed by aspiration, and the cell pellets were flash frozen on dry ice and stored at −80 °C until RNA preparation. Total RNA was isolated from frozen cells by modification of the 1step method (Chomczynski and Sacchi, 1987). The RNA pellet was resuspended in diethyl pyrocarbonate-treated water. Optical densities were measured at 260 and 280 nm, and integrity of RNA was visualized by subjecting 2–5 µl of the sample to electrophoresis through a 1% agarose-MOPS gel. Poly(A)$^+$ RNA was extracted using an Oligotex mRNA kit (Qiagen, Germany) and quantified using a RiboGreen RNA quantification kit (Molecular Probes, Eugene, OR). The fluorescent cDNA probes were prepared through reverse transcription with Cy3- or Cy5-deoxy UTP (Amersham Pharmacia Biotech, Piscataway, NJ) as follows: 5 µg of oligo (dT) 18 was added and annealed to 3 µg RNA by heating the reaction to 70 °C for 10 min, and then chilling on ice. The final reaction buffer mixture contained dNTPs (200 µM dATP, dCTP, dGTP, 60 µM dTTP, and 60 µM Cy3- or Cy5-dUTP), 2 µl Superscript II reverse transcriptase (Invitrogen), and $1 \times$ reaction buffer. Reactions were carried out at 42 °C for 2 h. RNA was hydrolyzed by adding 4 µl 2.5 M NaOH and incubating at 65 °C for 10 min and then neutralized with 4 µl 2.5 M HCl. The RNA samples from control group cells were labeled with Cy3-dUTP and those from experimental group cells with Cy5-dUTP. The 2 color probes were then mixed and diluted to 500 µl with TE buffer, and concentrated using a Microcon YM-30 filter (Millipore, Bedford, MA) to 10 µl. The sample was then vacuum dried.

Microarray hybridization

Each set of samples was used for separate array hybridizations. In the first two hybridizations cDNA from the susceptible isolate labeled with Cy3 and cDNA from the resistant isolate labeled with Cy5 was used, and in the third hybridization cDNA from the susceptible isolate labeled with Cy5 and cDNA from the resistant isolate labeled with Cy3. Thus, a total of three independent hybridization experiments were performed for each set of susceptible and resistant isolates. The fluorescently labeled cDNA probes were dissolved in 20 µl hybridization solution ($5 \times$ SSC, 0.4%SDS, 50% formamide). Microarrays were pre-hybridized with hybridization solution containing 0.5 mg/ml denatured salmon sperm DNA at 42 °C for 6 h. Fluorescent probe mixtures were denatured at 95 °C for 5 min, and the denatured probe mixtures were applied onto the pre-hybridized chip under a cover glass. Chips were

hybridized in a homemade chamber at 42 °C for 15–17 h. The hybridized chips were then washed at 60 °C for 10 min each in solutions of 2 × SSC and 0.2% SDS, 0.1 × SSC and 0.2% SDS and 0.1 × SSC, then dried at room temperature.

Detection and data analysis

The chips were scanned with a ScanArray 3000 (GSI Lumonics, Bellerica, MA) at 2 wavelengths to detect emission from both Cy3 and Cy5. GenePix 1.0 software (Axon Instruments, Inc.) was used for image analysis and data visualization. The intensities of each element at the 2 wavelengths represent the quantity of Cy3-dUTP and Cy5-dUTP, respectively, hybridized to each element. The local background values were calculated from the area surrounding each element and the background values were subtracted from the total element signal values. These adjusted values were used to determine differential gene expression (Cy5/Cy3 ratio) for each element. To rule out possible artifacts arising from low expression values, only genes with raw intensity values for both Cy3 and Cy5 of more than 800 counts were chosen for differential analysis (Li et al., 2002). To balance the systematic differences in the probe labeling or detection efficiencies between the fluorescent dyes used, a normalized factor N_{total} is calculated by summing the measured intensities in both channels

$$N_{\text{total}} = \frac{\sum_{i=1}^{N_{\text{array}}} Cy5_i}{\sum_{i=1}^{N_{\text{array}}} Cy3_i},$$

where $Cy5_i$ and $Cy3_i$ are the measured intensities for the ith array element and N_{array} is the total number of elements represented in the microarray. $Cy3$ intensities for each element are appropriately scaled,

$$Cy3'_k = N_{\text{total}} Cy3 \text{ and } Cy5'_k = Cy5,$$

so that the normalized expression ratio for each element becomes

$$\text{Ratio}_k = Cy5_k / Cy3'_k.$$

In the present study, only elements with a balanced differential expression ratio greater than or equal to 1.5 or less than or equal to 0.67 (−1.5) in all three independent experiments, were considered to be differentially expressed.

DNA sequences were annotated based on the results of BLASTN and BLASTX searches using GenBank (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi), the Stanford University (Palo Alto, CA) sequencing database (http://www-sequence.stanford.edu/group/candida/), and the CandidaDB database (http://genolist.pasteur.fr/CandidaDB/).

cDNA synthesis and reverse transcription-PCR (RT-PCR)

One μg of total RNA was used as a template for cDNA synthesis and subsequent RT-PCR amplification was performed using the One Step RNA PCR kit (AMV) from Takara following the manufacturer's instructions. The following parameters were used for the amplification of specific genes: reverse transcription at 50 °C for 30 min; heat inactivation of reverse transcriptase at 94 °C for 5 min; 26 to 34 PCR cycles (94 °C for 30 s, a gene-specific annealing temperature for 30 s, 72 °C for 90 s); and 72 °C for 5 min. Equivalent volumes of PCR products were applied to a 1.5% agarose gel with ethidium bromide and separated by gel electrophoresis in 1 × TAE. Primer sequences used for amplification of specific genes by RT-PCR are shown in Table 2.

Results and discussion

Differential gene expression in fluconazole-susceptible and -resistant isolates

The use of *C. albicans* microarray techniques permits the investigation of global changes in gene expression between drug-susceptible and drug-resistant isolates. TL1/TL3 and FH1/FH5, the two sets of clinical fluconazole-susceptible and -resistant isolates used in the present study have been well characterized and matched (Xu et al., 2005). FH1/FH5 were isolated from a patient who underwent a bone marrow transplant (BMT) operation and represent the same strain according to the identical banding patterns of the restriction fragment length polymorphism (RFLP) analysis. TL1/TL3 was obtained from another BMT patient, and revealed identical banding patterns in RFLP analysis. We examined a large-scale gene expression between TL1 and TL3, FH1 and FH5 simultaneously by the cDNA microarray containing 3096 unigenes in *C. albicans*. A total of 198 genes were found to be differentially expressed between TL3 and TL1 isolates, of which 105 genes were up-regulated and 93 genes were down-regulated (Table 3); and 257 differentially expressed genes between FH5 and FH1 isolates, including 110 up-regulated genes and 147 down-regulated genes (Table 4). The distribution of those genes and their biological roles are listed in the Supplementary Table in the online version of this article. The category of genes with the largest number of responses was that of unknown function (31.4%), followed by metabolism (24.4%), protein synthesis (11.0%), small-molecule transport (9.7%), amino acid metabolism (7.0%), cell stress (5.0%), transcription (3.5%), lipid, fatty acid, and sterol metabolism (2.3%), and others (5.7%) (Xu et al., 2005).

To confirm the differential expression result obtained by microarray, selective important *C. albicans* genes

Table 2. Sequences and melting temperatures (*Tm*) of primers used in RT-PCR[a]

Primer	Sequence	*Tm*(°C)
CaCDR1-F	5′-GGCAATTAGTCAAGACTCTTCTTCAG-3′	57.2
CaCDR1-R	5′-CACCTGGTCTCATAATGGCATC-3′	56.7
CaCDR2-F	5′-CAGCTAGACGAAAAGCCATGG-3′	57.1
CaCDR2-R	5′-CACCTGGTCTCATAATGGCATC-3′	56.7
CaIFU5-F	5′-TGAAGACAAAGAAATCCCAATG-3′	57.6
CaIFU5-R	5′-AATAGCATACCTCCTAACAAACC-3′	57.2
CaPDR16-F	5′-AATTCCTACAGGAGGGATTTTCC-3′	60.0
CaPDR16-R	5′-ATATTCCGTTATTTGCGAGCTTA-3′	58.7
CaIFD6-F	5′-AACTAAGTCTTCAGCATGAGCCAATT-3′	62.5
CaIFD6-R	5′-GGGCGTGTTTATGTCGTCCATT-3′	63.6
CaRTA2-F	5′-GACGATGACCTCTTACCGATTT-3′	57.8
CaRTA2-R	5′-CTTTCTGTGCGTCAAGTAACCA-3′	58.2
IPF7530-F	5′-CTATTGGAGAAGGCGAGGAAGA-3′	60.5
IPF7530-R	5′-TGTTGAGTCACATAGGCAGAAA-3′	55.9
CaYOR1-F	5′-AACCTCGGAATAATACTGGATCT-3′	56.3
CaYOR1-R	5′-AATGCTTCATTAGCTTATGAGACC-3′	57.3
CaPXA1-F	5′-ATCTTTGTCTTTCACCACCACTC-3′	57.6
CaPXA1-R	5′-ACGAGACCAAATAATTTACCCTC-3′	56.8
IPF10565-F	5′-CGGTAATCCTGATACTCTTGAA-3′	54.3
IPF10565-R	5′-AACTACTGATGGGAAACATAGA-3′	51.2
CaALD5-F	5′-TTGGTGGGTTGATTTTCATTCT-3′	58.8
CaALD5-R	5′-CTTTGGTCCAGTTGTCACTTTG-3′	57.4
CaSOD2-F	5′-AGCACTATCGG AAGTAACTC-3′	62.0
CaSOD2-R	5′-GTGGTTACCTCAATCATTGT-3′	62.0
CaGRP1-F	5′-TAGCATTATTGTTCACCCTTGC-3′	57.6
CaGRP1-R	5′-AGTGGTTGTAAACCCATCTTGG-3′	58.4
IPF8374-F	5′-CCCGAAAGTTTGTCCATGTTGT-3′	61.4
IPF8374-R	5′-GGCTTTATTTGCTGGTGGTAGA-3′	59.1
CaCCC2-F	5′-TATCATTTCCCATCGCCACCC-3′	64.2
CaCCC2-R	5′-CAGGGATTCAGGCTTTCTTCT-3′	57.9
CaCRD1-F	5′-AATTCAAAGCCATACTACTCAAGAC-3′	56.6
CaCRD1-R	5′-TGGGTGGTAATAAAGAACCAAGT-3′	57.8
CaCRD2-F	5′-TATAATAATTAAGGGGGGAGAGG-3′	56.5
CaCRD2-R	5′-ATGTTGATGATACTGAGGAGGGA-3′	57.9
CaCTR1-F	5′-GTCATAGCAGCCAACATCAAGG-3′	59.9
CaCTR1-R	5′-ATTATGCCGAAGTAGAACAAGG-3′	56.2
CaCTR2-F	5′-TATCGTTGAAGCATTGGAAGC-3′	58.2
CaCTR2-R	5′-TGGTGGCATGTTAAGACAGAA-3′	56.4
CaFET3-F	5′-GTGTTGCTGCGTTCTTAGGCT-3′	59.9
CaFET3-R	5′-CCATTTGGAATTTCACGGTTG-3′	60.4
18S rRNA-F	5′-GCCAGCGAGTATAAGGCTTG-3′	56.7
18S rRNA-R	5′-AGGCCTCACTAAGCCATTCA-3′	57.3

[a]Abbreviations: F, forward primer; R, reverse primer.

were subjected to RT-PCR to compare their transcription level. The results are shown in Figs. 1-3.

Identification of differentially expressed ATP-binding cassette (ABC) transporter superfamily genes

Expression of several ABC transporter genes was highly coordinated with resistant phenotype. The two ABC transporter genes, *CaCDR1* and *CaCDR2*, are known to be differentially expressed in many azole-resistant isolates and can be induced by various drugs, such as estradiol, fluphenazine and fluconazole (Franz et al., 1998; Karababa et al., 2004; Rex et al., 1995). For the two sets of isolates in the present study, it was shown that the resistant isolates TL3 and FH5 contained approximately 7- and 2.5-fold more mRNA for *CDR* (common probe for the *CaCDR1* and *CaCDR2*) by Northern analyses (Marr et al., 1998, 2001). Our microarray results also proved the up-regulation of *CaCDR1* in the resistant isolates, which was found to be

Table 3. Genes that were differentially expressed in TL3 compared to TL1 (partial)

Gene group and CandidaDB entry no.	ORF (Assembly 19)	Function (gene)[a]	Average fold change[b]
ABC transporters			
CA4190	*orf19.4531*	ABC protein (*IPF7530*)	+4.5
CA6066	*orf19.6000*	Multidrug resistance protein (*CaCDR1*)	+2.3
CA5844	*orf19.7500*	Long chain fatty acid ABC transporter (*CaPXA1*)	+2.1
CA6194	*orf19.1784*	ABC transporter protein (*CaYOR1*)	+1.8
Copper transporters			
CA2832	*orf19.4784*	Copper transporting P1-type ATPase (*CaCRD1*)	+3.3
AF268099[c]	—	Copper-binding metallothioneins (*CaCRD2*)	+2.8
CA1600	*orf19.4720*	Copper transport protein (*CaCTR2*)	+1.9
CA1655	*orf19.4328*	Copper-transporting P-type ATPase (*CaCCC2*)	+1.9
AF193509[c]	—	Copper resistance-associated metallothioneins (*CaCUP1*)	−2.6
CA4932	*orf19.6073*	Potential heme oxygenase, similar to *S. cerevisiae HMX1* (*IPF8374*)	−2.6
CA1496	*orf19.3646*	Copper transport protein (*CaCTR1*)	−3.8
Response to stress			
CA0281	*orf19.1438*	Potential oxidoreductase (*IPF10565*)	+3.6
CA0473	*orf19.4781*	Dihydroflavonol-4-reductases (*CaGRP1*)	+3.5
CA0917	*orf19.2969*	Nucleotide excision repair protein (*CaRAD16*)	+2.3
CA2719	*orf19.3340*	Manganese-superoxide dismutase (*CaSOD2*)	−1.7
CA4474	*orf19.1896*	Mitochondrial heat shock protein 70-related protein (*CaSSC1*)	−1.7
CA4347	*orf19.6722*	Excision repair protein, similar to *S. cerevisiae* Rad4p (*IPF3636*)	−2.0
CA3011	*orf19.6229*	Catalase A, peroxisomal (*CaCTA1*)	−2.2
CA4159	*orf19.5806*	Aldehyde dehydrogenase (NAD) (*CaALD5*)	−2.3
CA2190	*orf19.4774*	Alternative oxidase (*CaAOX1*)	−2.6
CA4034	*orf19.3664*	Heat shock protein (*CaHSP31*)	−2.7
CA2189	*orf19.4773*	Alternative oxidase (*CaAOX2*)	−4.4
Resistance related			
CA2679	*orf19.2568*	Unknown function (CaIFU5)	+7.8
CA4484	*orf19.1880*	Ferrochelatase precursor (*CaHEM15*)	+3.0
396066A06.sl.seq[d]	*orf*19.8629	Involved in lipid biosynthesis and multidrug resistance, similar to *S. cerevisiae* Pdr16p (*CaPDR16*)	+2.2
CA1943	*orf19.4737*	multidrug resistance proteins, similarity to *S. cerevisiae* Polyamine transport protein Ypr156c (*IPF11759*)	+2.1
CA2417	*orf19.4476*	Putative aryl-alcohol dehydrogenase (*CaIFD6*)	+1.8
CA0474	*orf19.4780*	Putative multidrug protein (*IPF12887*)	+1.7
CA4844	*orf19.3812*	Drug resistance (*CaPDR13*)	−1.6
CA1983	*orf19.88*	Ketol-acid reducto-isomerase (*CaILV5*)	−1.7

[a]Gene functions were obtained using the CandidaDB databases available from the Pasteur Institute.

[b]Response is expressed as the average fold change relative to the value for the susceptible isolate from three independent experiments. Values $> +1.5$ mean that the genes were up-regulated in resistant isolates versus the sensitive strains; values < -1.5 mean that the genes were down-regulated in resistant isolates versus the sensitive strains.

[c]Genes were identified at http://www.ncbi.nlm.nih.gov/entrez/query.fcgi.

[d]*PDR16* were identified at http://www-sequence.stanford.edu:8080/bin/blastnComb.

2.3- and 2.5-fold up-regulated in isolates TL3 and FH5, respectively. However, the *CaCDR2* gene was not represented on the array used in the present study. Therefore, to confirm the differential expression of *CaCDR1* and *CaCDR2*, independent RT-PCR experiments were performed to validate the up-regulation of *CaCDR1* and *CaCDR2* in the resistant isolates TL3 and FH5 (Fig. 1).

Besides *CaCDR1* and *CaCDR2*, we identified three other up-regulated ABC transporter genes, including

Table 4. Genes that were differentially expressed in FH5 compared to FH1 (partial)

Gene group and CandidaDB entry no.	ORF (Assembly 19)	Function[a]	Average fold change[b]
ABC transporters			
CA6066	*orf19.6000*	Multidrug resistance protein (*CaCDR1*)	+2.5
Copper transporters			
AF268099[c]	—	Copper-binding metallothioneins (*CaCRD2*)	+1.7
Y09329[c]	*orf19.4213*	Multicopper oxidase (*CaFET3*)	−2.1
CA1262	*orf19.1244*	Mac1p interacting protein (*CaGYP2*)	−2.1
CA4932	*orf19.6073*	Potential heme oxygenase, similar to *S. cerevisiae HMX1* (*IPF8374*)	−2.8
Response to stress			
CA0281	*orf19.1438*	Potential oxidoreductase (*IPF10565*)	+4.4
CA4034	*orf19.3664*	Heat shock protein (*CaHSP31*)	+3.0
CA1239	*orf19.717*	Heat Shock Protein 60 (*CaHSP60*)	+2.3
CA2190	*orf19.4774*	Alternative oxidase (*CaAOX1*)	+1.6
CA2857	*orf19.1065*	Heat shock protein of HSP70 family (*CaSSA1*)	−1.8
CA2719	*orf19.3340*	Manganese-superoxide dismutase (*CaSOD2*)	−1.9
CA4159	*orf19.5806*	Aldehyde dehydrogenase (NAD) (*CaALD5*)	−2.0
Resistance related			
CA2679	*orf19.2568*	Unknown function (CaIFU5)	+3.7
CA4696	*orf19.6924*	Histone H2A (*CaHTA1*)	+2.8
CA4807	*orf19.2094*	Pleiotropic drug resistance regulatory protein (*CaPDR6*)	+2.3
396066A06.sl.seq[d]	*orf19.8629*	Involved in lipid biosynthesis and multidrug resistance (*CaPDR16*)	+2.3
CA3607	*orf19.24*	Unknown function (CaRTA2)	+2.1
CA5932	*orf19.6771*	Polyubiquitin (*CaUBI4*)	−1.5
CA4844	*orf19.3812*	Drug resistance (*CaPDR13*)	−1.7
CA1959	*orf19.771*	Putative aryl-alcohol dehydrogenase (*IFD2*)	−1.8
CA1983	*orf19.88*	Ketol-acid reducto-isomerase (*CaILV5*)	−2.1

[a]Gene functions were obtained using the CandidaDB databases available from the Pasteur Institute.
[b]Response is expressed as the average fold change relative to the value for the susceptible isolate from three independent experiments.
[c]Genes were identified at http://www.ncbi.nlm.nih.gov/entrez/query.fcgi.
[d]*PDR16* were identified at http://www-sequence.stanford.edu:8080/bin/blastnComb.

IPF7530, *CaYOR1* and *CaPXA1*. *IPF7530* represented a putative membrane protein with 13 membrane-spanning domains, which was similar to *Saccharomyces cerevisiae* ABC protein YOL075c. Yor1p, a plasma membrane transporter of the ABC family, mediated export of many different organic anions including oligomycin (Karababa et al., 2004). Pxa1p appeared to be a subunit of a peroxisomal ABC transporter necessary for transport of long-chain fatty acids into peroxisomes. *IPF7530*, *CaYOR1* and *CaPXA1* were found to be up-regulated 4.5-, 1.8- and 2.1-fold in the resistant isolate TL3 as compared to the susceptible one TL1, respectively.

We have not found a significant change in *CaMDR1* between TL3 and TL1 or between FH5 and FH1 in our microarray results, and this is consistent with the previous observation (Marr et al., 1998, 2001).

Drug response element (DRE) of resistance-related genes and potential targets of *TAC1* regulation

Recently, the molecular mechanisms governing the regulation of multi-drug transporter genes have made some progress due to the discovery of *TAC1* in *C. albicans*. *TAC1* appeared to be the first *C. albicans* transcription factor involved in the control of genes mediating antifungal resistance. *TAC1* regulated the expression of *CaCDR1* and *CaCDR2*, and the deletion of *TAC1* can result in a loss of transient *CaCDR1* and *CaCDR2* up-regulation after drug exposure (Coste et al., 2004). A fusion protein of Tac1p with glutathione S-transferase could bind to the cis-acting regulatory drug-responsive element (DRE) in both the *CaCDR1* and *CaCDR2* promoters (Coste et al., 2004). The DRE sequence containing two 6-bp repetitive elements with

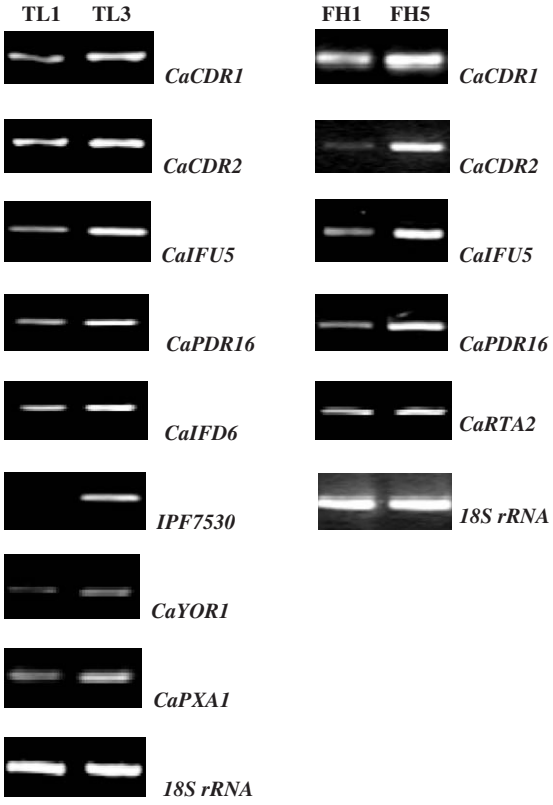

Fig. 1. Evaluation of differential expression by RT-PCR of ABC transporter genes and potential target genes of *TAC1* found to be associated with azole antifungal resistance in isolates TL1/TL3 and/or FH1/FH5. RT-PCR of *C. albicans* 18S rRNA was performed as a control.

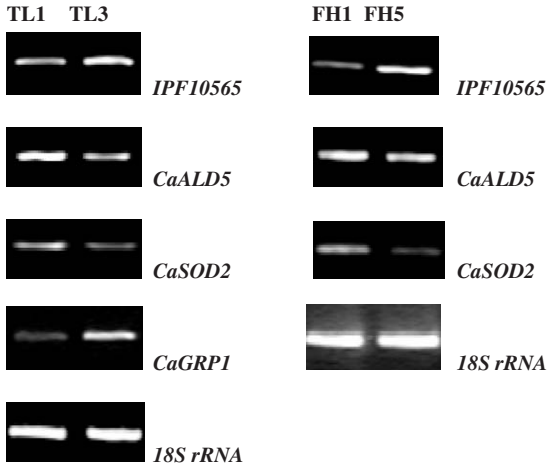

Fig. 2. Evaluation of differential expression by RT-PCR of oxidative stress response genes found to be associated with azole antifungal resistance in isolates TL1/TL3 and/or FH1/FH5. RT-PCR of *C. albicans* 18S rRNA was performed as a control.

5′-CGG-3′ triplets is known to be required for the basal activity of *CaCDR1* and the up-regulation of *CaCDR1* and *CaCDR2* (de Micheli et al., 2002). Besides *CaCDR1*

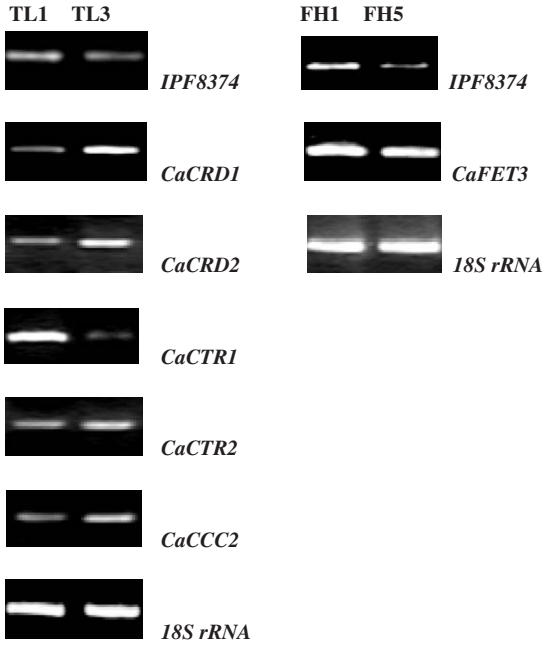

Fig. 3. Evaluation of differential expression by RT-PCR of copper and iron transport genes found to be associated with azole antifungal resistance in isolates TL1/TL3 and/or FH1/FH5. RT-PCR of *C. albicans* 18S rRNA was performed as a control.

and *CaCDR2*, three other genes *CaIFU5*, *CaRTA3* and *CaHSP12* that contained a DRE-like element in their promoters, were also identified as targets of *TAC1* regulation (Coste et al., 2004).

It is reported that 15 over-expressed genes activated by the *PDR1-3* and *PDR3-7* mutations in *S. cerevisiae* contained a PDRE (pleiotropic drug-responsive element) element (TCGG/aC/tGG/cA/g, where lowercase letters indicate variable bases in a consensus motif) in their promoters (DeRisi et al., 2000). Interestingly, for seven of the fifteen genes, their *C. albicans* homologues showed differential expression in the present study (Table 5). Furthermore, four of these genes including *CaCDR1*, *CaIFU5*, *CaPDR16* and *CaIFD6* were found to have a DRE-like element (CGGWWWTCGGWWW) in their promoters, while the other four genes (*CaRTA2*, *CaIFD2*, *CaYOR1*, *CaGRP1*) were found to have a PDRE element in their *S. cerevisiae* homologues and they do not contain DRE-like elements in the promoters (Table 5).

For two predicted membrane proteins, *CaIFU5* and *CaRTA3*, increased transcription has been demonstrated under conditions of *CDR* overexpression or fluphenazine treatment (Karababa et al., 2004). Although *CaRTA3* was not represented on the array used in the present study, we have observed the differential expression of *CaIFU5* and *CaRTA2*. *CaIFU5* was up-regulated 7.8- and 3.7-fold in both resistant

Table 5. Genes containing DRE or PDRE sequences with differential expression in the present study

Gene name	S. cerevisiae homologue name	Function[a]	S. cerevisiae homologue with PDRE[b]	Possible DRE[c] sequence	Average fold change	
					TL3 vs TL1	FH5 vs FH1
CA6066	*PDR5/YOR153w*	Multidrug resistance protein (*CaCDR1*)	TCCGtGGA	**CGGAAATCGG**ATA (−460)	+2.3	+2.5
CA2679	YFL010c	Unknown function (CaIFU5)	No PDRE	**CGGAAATCGG**ATA (−248)	+7.8	+3.7
396066A06.sl.seq	*YNL231c*	Involved in lipid biosynthesis and multidrug resistance (*CaPDR16*)	TCCGCGGA	**CGGATTCGG**AAT (−562)	+2.2	+2.3
CA2417	YPL088w	Putative aryl-alcohol dehydrogenase (*CaIFD6*)	TCCaCGGA	**CGG**TTg**TCGG**AAT (−352)	+1.8	No difference[d]
CA3607	YOR049c	Unknown function (CaRTA2)	TCCGCGGA	No DRE	No difference	+2.1
CA1959	YPL088w	Putative aryl-alcohol dehydrogenase (*CaIFD2*)	TCCaCGGA	No DRE	No difference	−1.8
CA6194	YGR281w	ABC transporter protein (*CaYOR1*)	TCCGtGGA	No DRE	+1.8	No difference
CA0473	GRE2/YOL151w	Stress-induced methylglyoxal reductase (*CaGRP1*)	TCCGtGGA	No DRE	+3.5	No difference

[a]Gene functions were obtained using the CandidaDB databases available from the Pasteur Institute.
[b]Bases indicated in lowercase type are mismatches compared to the following generic PDRE sequence: TCCG/aC/tGG/cA/g.
[c]Bases indicated in lowercase type are mismatches compared to the following generic DRE sequence: CGGWWWTCGGWWW. Position of the DRE relative to the start codon is in parentheses.
[d]No difference indicated having no differential expression between the resistant and susceptible isolates.

isolates TL3 and FH5, respectively, and *CaRTA2* was up-regulated 2.1-fold in the resistant isolate FH5. The function of *CaIFU5* remains unknown. *CaRTA2* shares the same *S. cerevisiae* homologue *YOR049c* with *CaRTA3*, but unlike *CaRTA3*, *CaRTA2* does not have a DRE-like element in its promoter. *YOR049c*, an ATP-dependent transporter or flippase with seven transmembrane-spanning domains, is involved in the resistance to sphingoid long-chain base (LCBs) (Kihara and Igarashi, 2002). Similarly, Barker et al. (2004) also reported the over-expression of *CaIFU5* and *CaRTA2* in the serially passaged resistant strain SC5314-AR, which was treated with increasing concentrations of amphotericin B.

We also found that *CaPDR16* was up-regulated 2.2- and 2.3-fold in both resistant isolates TL3 and FH5, respectively. *CaPDR16* encodes a phosphatidylinositol transfer protein, which modifies the passive diffusion of hydrophobic drugs across the plasma membrane, thereby modulating multi-drug resistance in *S. cerevisiae* (van den Hazel et al., 1999). Recently it was found that PDR16 was co-expressed with *CaCDR1* and *CaCDR2* in clinical resistant strain 5674 or upon fluphenazine treatment (de Deken and Raymond, 2004). *CaPDR16* not only has a PDRE element in its *S. cerevisiae* homologue, which was up-regulated in the *PDR1-3* and *PDR3-7* mutants, but also has a DRE-like element in the promoters though one mismatch was found as compared with the consensus sequence (Table 5).

CaIFD6 was 1.8-fold up-regulated in the resistant isolate TL3. *CaIFD6* has a DRE-like motif in its promoter though one mismatch was found as compared with the consensus sequence (Table 5), thus indicating that its over-expression might be potentially regulated by *TAC1*. However, *CaIFD2* had no DRE element and also showed 1.8-fold down-regulation in the resistant isolate FH5. *CaIFD6* and *CaIFD2* are two members of the *IFD* family which is homologous to *YPL088W* in *S. cerevisiae*, a family of putative alcohol dehydrogenase/oxidoreductase. *YPL088W* has a PDRE element in the promoter and it is one of the 26 genes upregulated in the pdr1-3 mutant strain in *S. cerevisiae*, indicating its responsiveness to *PDR1* and *PDR3* (DeRisi et al., 2000). Members of the *IFD* gene family, the encoded products of which could have benzyl alcohol dehydrogenase activity, especially *CaIFD1*, *CaIFD4*, *CaIFD5*, and *CaIFD7*, have been found to be over-expressed in multiple resistant isolates, including clinical isolates with stepwise resistance from an OPC patient with AIDS, clinical isolates with *CaMDR1* up-regulation, and resistant strains treated with benomyl or H_2O_2 (Rogers and Barker, 2002, 2003; Karababa et al., 2004). *CaIFD6* and *CaIFD2* have also been observed to be up-regulated in two proteomic studies comparing susceptible and resistant *C. albicans* isolates (Hooshdaran et al., 2004; Kusch et al., 2004).

Role of oxidative stress response genes

IPF10565 was found to be up-regulated 4.3- and 3.1-fold in both isolates TL3 and FH5, respectively. *IPF10565* is a potential oxidoreductase that is similar to *CPD1* in *S. cerevisiae*. *ScCPD1* encodes a protein of the FAD-dependent pyridine nucleotide reductases. Over-expression of either of Cpd1p and Snq2p could mediate resistance to singlet oxygen-generating compounds (Ververidis et al., 2001). Snq2p is a well-characterized ABC-type multi-drug efflux protein in *S. cerevisiae* and its direct role in multi-drug and steroid export has been previously established (Anderson et al., 2003).

CaALD5 was down-regulated 2.3- and 2.0-fold in both resistant isolates TL3 and FH5 from the BMT patients, respectively. *CaALD5* encodes mitochondrial aldehyde dehydrogenase which plays a role in regulation or biosynthesis of electron transport chain components (Kurita and Nishida, 1999). Impairment or loss of mitochondrial function was reported to be associated with increased expression of Pdr5p in *S. cerevisiae* (Hallstrom and Moye-Rowley, 2000). Since the ald5 mutant is defective in respiration, downregulation of *CaALD5* may therefore contribute to overexpression of *CaCDR1* and *CaCDR2* in resistant isolates. Previous studies have shown a relationship between *CaALD5* and azole resistance in *C. albicans* from an OPC patient with AIDS, as *CaALD5* was down-regulated 3.8-fold in resistant isolate 2–79 to 12–99 (Rogers and Barker, 2002).

We observed that *CaGRP1* was also up-regulated 3.9-fold in the resistant isolates TL3. *CaGRP1* is a homolog of *GRE2* in *S. cerevisiae* which encodes a putative reductase similar to plant dihydroflavonol-4-reductases, and it responses to osmotic stress, ionic, oxidative, heat stress and heavy metals (Rogers and Barker, 2003). Containing a PDRE element in the promoter, ScGre2p has been demonstrated to be up-regulated in azole resistant *S. cerevisiae* isolates with constitutive activation of the transcription factor PDR3 (DeRisi et al., 2000; Nawrocki et al., 2001). In *C. albicans*, *CaGRP2* was found to be up-regulated in the stepwise resistance development from susceptible to resistant isolates by microarray and proteomic studies (Hooshdaran et al., 2004; Rogers and Barker, 2003), and transcriptional increased upon fluphenazine or benomyl treatment (Karababa et al., 2004).

CaSOD2 was down-regulated 1.7- and 1.9-fold in the resistant isolate TL3 and FH5, respectively. The downstream gene *CaCTA1* of *CaSOD2* was also down-regulated in resistant isolate TL3. Superoxide dismutases (SODs) convert superoxide radicals into less damaging hydrogen peroxide, and the catalase encoded by *CaCTA1* then converts hydrogen peroxide to water (Martchenko et al., 2004). *CaSOD1*, encoding the

cytosolic copper- and zinc-containing SOD (CuZn-SOD), is required for the protection against oxidative stresses and the establishment of full virulence (Hwang et al., 2002). The down-regulation of *CaSOD1* has been observed in multiple resistant isolates in *C. albicans*, including the isolates treated by benomyl, fluconazole and a series of isolates with stepwise resistance from a clinical OPC patient with AIDS (Cowen et al., 2002; Karababa et al., 2004; Rogers and Barker, 2003). *C. albicans CaSOD2* encoded a mitochondrial manganese-containing SOD (MnSOD), which had no effect on the virulence aspect of this fungus but protects the cell against intracellularly produced superoxides.

Copper transport and iron mobilization related genes

One of our important findings is the observation of the differential expression of a series of copper and iron transporter genes. Copper is essential for enzymes involved in a multitude of biological processes such as signaling the transcription and protein trafficking machinery, oxidative phosphorylation, iron mobilization, neuropeptide maturation, and normal development (Rees et al., 2004; Riggle and Kumamoto, 2000). The acquisition of iron, which is an equally important factor, has also been known to be important for the growth, survival and virulence of *C. albicans* (Ramanan and Wang, 2000). The organism can acquire iron from heme and establish an infection by binding to and lysing erythrocytes (Marvin et al., 2003). Interestingly, we have observed a series of copper and iron mobilization-related genes which showed differential expression between the two sets of resistant and susceptible isolates. From our results, *CaCRD1*, *CaCRD2*, *CaCTR2* and *CaCCC2* were all up-regulated in the resistant isolates TL3 and/or FH5, whereas *CaCTR1*, *CaFET3*, *CaCUP1* and *CaGYP2* were down-regulated in the resistant isolates TL3 and/or FH5.

CaCRD1 and *CaCRD2* encode P1-type ATPases as copper-binding metallothioneins. *CaCRD1* is mainly involved in high-level copper resistance in *C. albicans* (Riggle and Kumamoto, 2000). In addition to its role in copper homeostasis Crd2p may also play a role in reducing oxidative stress (Palmiter, 1998; Tamai et al., 1993). We observed a 3.5-fold up-regulation of *CaCRD1* in the resistant isolate TL3 while *CaCRD2* was up-regulated 2.8- and 1.7-fold in both resistant isolates, TL3 and FH5, respectively. This up-regulation can lead to copper resistance in the resistant isolates.

The multi-copper oxidase gene *CaFET3* and the copper transporter *CaCCC2* are both required for high-affinity iron import (Marvin et al., 2004; Ramanan and Wang, 2000; Weissman et al., 2002). A homozygous deletion of *CaCCC2* resulted in strains with defective high-affinity iron uptake, presumably as a result of incorrect delivery and incorporation of copper into CaFet3p (Marvin et al., 2004). We found that *CaCCC2* was up-regulated 1.9-fold in TL3 and *CaFET3* was down-regulated 2.1-fold in the resistant isolate FH5, as compared to thei susceptible isolate.

Recent research pointed out that the absence of heme resulted in decreased transcription of genes belonging to both the iron and copper regulons, and proved that the inhibition of heme biosynthesis can prevent transcription of iron uptake genes in yeast (Crisp et al., 2003). In our study, *IPF8374* showed a 2.8- and 3.1-fold down-regulation in resistant isolates TL3 and FH5, respectively. *IPF8374* is similar to *HMX1* in *S. cerevisiae*, which encodes a potential heme oxygenase. It catalyzes the rate-limiting step in the degradation of heme to bilirubin, and it is essential for recycling of iron from heme. Deletion of *HMX1* resulted in a decrease in heme degradation activity, so it is important for heme iron reutilization and the homeostasis of regulatory pools of iron and heme (Protchenko and Philpott, 2003). It is reasonable to presume that down-regulation of *IPF8374* resulted in a decrease of heme degradation activity. If heme is increased, there may be not enough fluconazole to bind to the excessive heme, resulting in an incomplete blocking of 14α-demethylase (CYP51). Our observation provides a possible link between excess heme and azole resistance in *C. albicans*.

CaCTR1 is required for high-affinity copper uptake in *C. albicans*. When heterologously expressed in *S. cerevisiae*, *CaCTR1* was highly expressed in conditions of copper limitation and strongly repressed in conditions of copper excess (Marvin et al., 2003). Our results showed that *CaCTR1* was down-regulated 3.8-fold in the resistant isolate TL3. It was consistent with its function of facilitating the transport of copper into the cell (Marvin et al., 2003), and also coincided with the up-regulated behavior of those copper efflux transporter *CaCCC2* and copper-binding metallothioneins *CaCRD1* and *CaCRD2* in the present study. Unlike *CaCTR1* encoding membrane-associated copper transport proteins, Ctr2p is localized to the vacuole membrane and provides bioavailable copper via mobilization of intracellular vacuolar copper stores in *S. cerevisiae*. We showed that *CaCTR2* was up-regulated 1.9-fold, and the over-expression of *CaCTR2* possibly led to the decrease of copper stores in the vacuole and its increase in the cytoplasm due to the transport of copper from vacuole into cytoplasm (Labbe et al., 1997).

Conclusions

To our knowledge, our study (this report and (Xu et al., 2005)) is the first to use cDNA microarray analysis to investigate large-scale gene expression in two sets of azole-resistant/-susceptible *C. albicans* isolates from

BMT patients. Our results demonstrate that the transcriptional profile of azole-resistant isolates from BMT patients is similar to that of isolates from OPC patients with increased expression of CDR mRNA. Some common genes involved in drug resistance, which have been demonstrated previously in other resistant clinical isolates or experimental strains, have also been observed in the present study, such as the up-regulation of *CaIFU5*, *CaRTA2*, *CaIFD6*, and *CaGRP1*. Some ABC transporter genes (*IPF7530*, *CaYOR1*, *CaPXA1*), oxidative stress response genes (*IPF10565*, *CaALD5*, *CaGRP1*, *CaSOD2*, *CaCTA1*), and copper transport and iron mobilization-related genes (*CaCRD1/2*, *CaCTR1/2*, *CaCCC2*, *CaCUP1*, *CaFET3*) were found to be associated with the resistant phenotype. Furthermore, some genes co-regulated with *CaCDR1*, *CaCDR2* and *CaIFU5*, such as *CaPDR16* and *CaIFD6*, have a DRE-like element and may interact with *TAC1* in the promoter region. Further studies are needed to investigate the specific contribution of these genes to the azole-resistant phenotype.

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Appendix A. Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ijmm.2006.03.004.

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